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Docket No.: 04287/100M315-US1
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
ARTHUR RAMAZANOV et al.

Application No.: 10/660,256 Art Unit: 1614

Filed: September 11, 2003 Examiner: Phyllis G. Spivack

For: A NOVEL COMPOSITION FOR THE TREATMENT OF OBESITY AND EFFECTIVE FAT LOSS PROMOTION

DECLARATION OF ZAKIR RAMAZANOV UNDER 37 C.F.R. § 1.132

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, ZAKIR RAMAZANOV, do hereby declare and state the following:

1. I am a citizen of the United States, and more than twenty-one years of age.

2. I make this declaration in support of the above-identified application ("the '256 application").

3. I am a co-inventor of the '256 Application, and have read the Office Action mailed November 17, 2005.

4. I presently hold the position of President & CEO of National Bioscience Corp., located in Warwick, NY (assignee of the present application) and have held this position for 6 years.

5. My qualifications as a scientist, and in particular in the field of neutraceuticals, are set forth on my *curriculum vitae* (copy attached as Exhibit A).

6. The '256 Application refers to aralosides that have an effect on weight, *i.e.* aralosides A, B, C, and D (*see* US2004/0147460, ¶¶ 0020; 0031; 0041; 0078; Fig. 2). Additionally, the Application also discloses aralosides extracted from *Aralia mandshurica/elata* ("*Aralia*") root bark in Example 1., ¶ 0067. The '256 Application is directed to compositions and methods for obesity control through treatment with aralosides in combination with a dihydroquercetin.

7. Paragraphs 8-14 below describe recent experiments conducted at my request and under my supervision and control.

8. An experiment was conducted under my supervision wherein HPLC comparative analysis of *Aralia* root bark and bud extracts was performed as set forth below.

Aralia root bark and *Aralia* buds were harvested in early August in Russia, sliced into 10-20mm particles, and dried in a drying chamber under warm airflow until the moisture content was reduced to about 8-15%. 10kgs of *Aralia* root bark particles were placed in a 100 liter extraction vessel and extracted with 100 liters ethanol: water at a 70:30 (volume/volume) mixture. Similarly, 10kgs of *Aralia* bud particles were placed in a 100 liter extraction vessel and extracted with 100 liters ethanol: water at a 70:30 (volume/volume) mixture.

The root bark material and the bud material were each combined with solvent in a separate vessel under agitation. Following this initial extraction, the remaining plant material in

each vessel was removed by filtration through a #40-mesh sieve and then subjected to a second extraction with an additional 10 liters of solvent. Following the second extraction, the residual plant material from each vessel was removed and discarded. The initial and secondary extracts were combined, and the alcohol evaporated under reduced pressure (about 250 bars) and elevated temperature (about 60-70°C). This alcohol-free solution was dried with a spray dryer.

The total concentration of aralosides for root bark and bud material was determined using the HPLC method described by Xu, et al.., Yao Xue Xue Bao, 33:933-936 (1998); the content of individual aralosides was determined using a Reversed-Phase High Performance Liquid Chromatographic (RP-HPLC) with Photodiode Array detection (Waters Alliance 2690 HPLC system, equipped with a 996 photodiode array detector). A Luna C18 column (150 x 4, 6 mm, 5µm particle size) was employed. Reference standard araloside material was purchased from the Russian Institute of Natural Products (Moscow). The resulting HPLC fingerprints of the *Aralia* root bark and bud extract are shown in annexed Exhibits B and C, respectively. The HPLC chromatogram of araloside A, used as reference standard, is shown in Exhibit D.

The HPLC comparative analysis depicted on Exhibits B, C, and D demonstrates that the saponin compositions of *Aralia* root bark and bud material are clearly different. Specifically, the major triterpene constituents in *Aralia* bud extract were oleanolic acid as major constituent, with triterpene saponins Congmuyanoside A and Congmuyanoside B as co-constituents (*see* Exhibit B). These results are in agreement with recent data of Ma Z-Q et al. (2005) Journal of Asian Natural Products Research, 7(6):817-21 (December 2005) (*see* Exhibit E). The major constituents in the *Aralia* root bark extract, by contrast, were aralosides A, B, C and D (*see* Exhibit C). Thus, *Aralia* root bark and *Aralia* bud extracts contain structurally different triterpene saponins; i.e., extracts derived from different vegetative parts of the same *Aralia* plant are phytochemically different. The physiologic effects of these differences are highlighted in the study described below in ¶¶ 9-14.

9. A double-blind, randomized, placebo-controlled study was conducted under my supervision. Body mass changes, fat mass loss, adipocyte perilipin content, and adipocyte perilipin synthesis were evaluated in patients treated with *Aralia* root bark extract ("ARE") or *Aralia* bud extract ("ABE"), each in combination with a dihydroquercetin. The methods and results of this study are described in the following paragraphs.

10. In order to conduct the study, ARE and ABE dosage forms were created as follows. To prepare the ARE dose, *Aralia* root bark extract (standardized to a minimum of 20% aralosides) was combined with the extract of *Engelhardtia chrysolepis* leaf (standardized to minimum 20% dihydroquercetin). Each 300mg ARE dose contained a powdered blend of active dried extract in a 1:1 ratio (i.e., 150mg each of *Aralia* root bark araloside and dihydroquercetin), formed into a tablet. Analogously, to prepare the ABE dose, *Aralia* bud extract (standardized to 20% oleanolic acids and triterpene saponins) was combined with the extract of *Engelhardtia chrysolepis* leaf (standardized to minimum 20% dihydroquercetin). Each 300mg ABE dose contained a powdered blend of active dried extract in a 1:1 ratio (i.e., 150mg each of *Aralia* bud araloside and dihydroquercetin), formed into a tablet. The Placebo dose were 300mg cellulose tablets.

11. Patients for the study were selected, treated, and monitored as follows. Selection criteria included the absence of clinically manifested diabetes, and no renal or thyroid disorders. All subjects taking medications known to influence lipid metabolism were excluded. An additional criterion for inclusion was that subjects were not engaged in physical exercise program. All potential volunteers underwent a medical examination, including routine laboratory testing and a 75g oral glucose tolerance test, prior to inclusion. Subjects meeting the criteria were randomly assigned to the ARE, ABE, or placebo group using the Simple Randomization Procedure. All patients were directed to take 300mg tablets of either (a) the ARE dose (n=15), (b) the ABE dose (n=15), or (c) the placebo

dose (n=15) three times a day before meals for 15 weeks. Daily dietary intake of study participants was restricted to 1700±150 kcals, 50% derived from carbohydrates, 25% from protein, and 25% from fat. Food record analyses, body composition, and blood and adipose biopsy samples were assessed for each subject on admission and after completion of the study. During the clinical phase subjects were weighed twice per week and questioned about compliance. Subjects were encouraged to maintain a normal lifestyle during the study, and all food and beverages were provided to subjects. Calorie distribution throughout the day was 30% for breakfast, 45-50% for lunch, and 20-25% for dinner. Subjects were instructed to consume all the food and beverages designated by dieticians and provided for them, and to eat no other food or high calorie beverages. To evaluate the accuracy of dietary nutrient intakes, the osmolar excretion rate technique was used, as described by Roberts S.B. et al., "Objective verification of dietary intake by measurement of urine osmolarity," *American. J. Clinic. Nutr.* 54:774-82 (1991). This method compares urine osmolar excretion rate and urine weight to the values for urine osmolar excretion rate predicted from dietary nitrogen, sodium, and potassium, using a micro-osmometer.

12. The study described above evaluated, *inter alia*, body weight and body fat mass changes. These variables were measured upon admission to the study (i.e., baseline values), and again at conclusion of the study 15 weeks later. Total body weight was measured in kilograms (kg). Percentage of body fat was then estimated from body density derived from underwater weighing. This percentage was used to calculate body fat mass (kg). Fat-free body mass was calculated by subtracting body fat mass from total body mass Siri, W.E., "The gross composition of body fat," *Adv. Biol. Med. Physiol.*, 4:239-80 (1956).

The following results were obtained from analysis of the participants in the study:

	ARE PARTICIPANTS	ABE PARTICIPANTS	PLACEBO PARTICIPANTS
WEIGHT BEFORE	95.5 ±3.7	94.6 ±3.3	95.9 ±3.6

WEIGHT AFTER	90.7 ±2.8	93.5 ±2.9	95.0 ±3.0
AVERAGE WEIGHT LOSS	4.8 ±0.4*	1.1 ±0.2	0.9 ±0.2
PERCENT WEIGHT LOSS	5.0%	1.2%	0.9%
WEIGHT REDUCTION DUE TO BODY FAT LOSS	4.5 ±0.7	0.8 ±0.3	0.7 ±0.2

All weights are in kilograms.
* ($p<.001$)

These results demonstrate that the body weight lost in the ARE group was preferentially due to body fat loss. These results also demonstrate that the body fat loss was specific to the ARE preparation, because the ABE and placebo dose groups did not experience fat loss effects.

13. The 15-week study described above also evaluated the effects of ARE and ABE treatment on human adipocytes total perilipin protein content. One day before beginning the study, and again at the end of the study, a subcutaneous fat biopsy was collected from each subject in the study. A cutaneous incision was made, and approximately 400–500mg of subcutaneous adipose tissue was surgically removed. Adipose tissue samples were homogenized using standard laboratory protocols to obtain adipocytes.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the adipocytes from each treatment group was performed. Levels of perilipin protein on isolated adipocytes were quantified using Western blot immunoelectrophoresis analysis. Adipocyte samples for SDS-PAGE electrophoresis were boiled, centrifuged, and the supernatant of each sample was removed. The protein concentration of the sample was determined by measuring absorbance in a spectrophotometer at 280nm. Supernatant aliquots containing 100µg of protein were separated on 10-20% linear gradient SDS-PAGE gels. Proteins were electrophoretically transferred onto

nitrocellulose membranes, placed in a blocking buffer, and subsequently incubated with polyclonal anti-perilipin. Membranes were then incubated with a secondary alkaline phosphatase-conjugated anti-rabbit IgG. Perilipin protein bound to the antibody was then detected using the alkaline phosphatase substrates nitroblue tetrazolium and bromo-chloro-indolyl phosphate. The relative perilipin protein concentration of each sample was determined by scanning densitometry (Hoefer GS-300 Scanning Densitometer). Scanning densitometry results were converted to arbitrary units for comparison. Results of this study are depicted in Exhibit F.

As can be seen in Exhibit F, the perilipin content was significantly reduced in adipocytes from patients in the ARE group, while there was no discernable change in the perilipin content of adipocytes taken from ABE or placebo group patients.

14. The 15 week study described above also evaluated the effects of ARE and ABE treatment on the perilipin protein synthesis rate in isolated adipocytes using the well-known ^{35}S-Methionine pulse–chase labeling method. Scheetz, A.J., et al., "NMDA receptor-mediated control of protein synthesis at developing synapses," *Nature Neuroscience* 3(3):211-16 (2000).

Adipose tissue obtained via biopsy before, and again after, the study from all three treatment groups (i.e., ARE, ABE, placebo) was prepared for ^{35}S-Methionine pulse–chase labeling. Samples were each separately incubated in chamber, and 50 μCi of ^{35}S-methionine was added to each sample. After incubation with the label, non-radioactive methionine was added. To stop protein synthesis, samples were treated with ice-cold 10% trichloroacetic acid, pelleted by centrifugation twice with washings between each spin. The resulting pellets were thereafter freeze dried. Dry samples were solubilized for separation using SDS-PAGE. The amount of radioactivity incorporated into adipocyte proteins was determined by placing sample aliquots in buffer and counting with a Beckman LS 1801 liquid scintillation counter. To compare radioactivity levels of the three different treatment groups, each sample was loaded to equal counts (150,000 count min^{-1} per lane).

After electrophoresis, the gels were dried and autoradiography was performed; radioactive ^{35}S-methionine incorporated proteins synthesized *de novo* in adipocytes were detected using X-ray film.

The results, shown in Exhibit G, demonstrate that before treatment there were no differences in the amount of 62-65kDa perilipin peptide between the three treatment groups. However, adipocytes from the ARE participants synthesized significantly less (27 ±7%) perilipin protein than did adipocytes from the participants in the ABE or placebo dose groups. Specifically, adipocytes from patients treated with the ARE dose synthesized approximately 27 ±7% less 62-65kDa perilipin polypeptide. There were no differences observed between pre-treatment and post-treatment adipocyte perilipin protein synthesis levels in adipocytes obtained from the ABE or placebo dose groups.

15. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true. I further declare that these statements are made with the knowledge that the willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States code, and that such willful false statements may jeopardize the validity of the instant application or of any patent issued thereupon.

Respectfully submitted,

March 12/06

DATE

Zakir Ramazanov

Curriculum Vitae
Professional Resume

Dr. Zakir Ramazanov

Warwick, NY 10990, USA

Name:	Zakir Ramazanov
Home Address:	22 Valley View Circle, Warwick, NY 10990, USA
Home Phone:	845-988-0118
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Office Address:	National Bioscience, 193 Black Meadow Road, Chester, NY 10918
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Corporate Web:	www.rhodiolarosea.org
Corporate Phone:	845-469-6143
Corporate Fax:	845-469-1983

Professional experience

2000-at present time:	President & CEO of National Bioscience Corporation, Warwick, NY 10990, USA
1996-1999	Executive Vise-President of Research and Development, Pharmline Inc., Florida NY 10951
1994 -1999	Director of Science & Professor of Research, at Institute of Applied Algology, University of Las Palmas, and the Technological Institute of Canary Islands, Development of commercial production of β-carotene, lycopene and lutein from tomato & algae, Large scale cultivation of unicellular algae Chlorella and blue-green algae Spirulina platensis, Cultivation and extraction of marine natural products Director of Ph D Programs at the Institute of Applied Algology, University of Las Palmas.
1991-1994	**Grant from US National Science Foundation** Louisiana State University, USA. Research Associate, USA, Biochemistry & Molecular Biology of CO_2 assimilation in the green algae. Isolation and characterization of high CO_2 requiring mutants of algae. Biotechnology of microorganisms.
1990-1991	**Grant from Spanish Government: Ministerio de Educacion i Ciencia.** Professor of Plant Biochemistry, University of Cordoba, Spain Intracellular mechanisms of Carbon and Nitrogen assimilation. Induction of carbonic anhydrase and nitrate reductase in aquatic plants; Intracellular localization of nitrogen assimilating enzymes in algae.
1989-1990	**Grant from Swedish Royal Academy and Institute S-90187** Post.Doctoral researcher, University of Umea, Sweden, Intracellular mechanisms of CO_2 assimilation by aquatic plants. Biochemistry of the cells adaptation to low and high CO_2 concentrations in the environments.
1986-1989	Chairman of Biotechnological programs development of Eastern European countries and Soviet Union. Scientific Director of the East-European programs for Biotechnology Development. Commercial cultivation of algae for human

consumption Space Biology & research on cultivation of photosynthetic micro-organisms on Russian Orbital Station "Saluyt".

1984-1989 — Senior Scientists, Chairman of Biotechnology Department, KA. Timiriazev Institute of Plant Physiology, the USSR Academy of Science. Construction of Photo-Bioreactors for the USSR Space Programs and East European Space Program "Inter-Cosmos".

1982-1984 — Graduate Student, the USSR Academy of Sciences, Institute of Plant Physiology, Ph. D. in Plant Physiology and Biochemistry. The title of Ph.D. Dissertation "Molecular Mechanisms of adaptation of photosynthetic organisms to high and low CO_2 concentrations".

1980-1981 — Head Engineer at the Institute of Solar Energy, The USSR Military and Aeronautic Industry. Design and Construction of Solar batteries for the USSR Military Service in the Middle Asian desert areas for potential use on Orbital Stations. Construction of Solar Bioreactors for cultivation of microorganisms in Closed Ecological systems, such as Orbital Stations, Cultivation of blue-green algae on Deuterium-enriched culture medium for the USSR Industry of Medicine.

PROFESSIONAL ACTIVITY

From 2000-	President of National Bioscience, Warwick, NY 10990, USA
1990-1998:	Member of Advanced Courses Committee of the European Society of Plant Physiologists (FESPP)
1990-1996:	Member of FESPP-AWARD Committee
1994-1996:	Director of Ph.D. Thesis of Miguel Jimenez del Rio, University of Palmas, Spain.
1994-1996:	Director of Ph. D. Thesis of Arsenio Villarejo, University of Autonoma de Madrid
1996:	Director of Ph. D. Thesis of Mtolera Mtern, University of Uppsala, Sweden, Marine Science Institute, Zanzibar
1995-1998:	Director of Ph. D. Thesis of Marie del Pino Plumed Tavio, University of Las Palmas, Spain

PATENTS

1987: Semenenko VE, Vladimirova MG and Ramazanov Z. (1987) New Chlorella vulgaris strain can be used as highly efficient producer of green biomass and vitamins. 09.08. SU- 1530637.

1989: Semenenko VE, Ramazanov Z. (1989) Production of β-carotene from Dunaliella salina cultivation. 03.08. SU-1513911

1991: Semenenko V, Loyde M., Loyde L., Ramazanov Z. (1991) Photobioreactor for commercial cultivation of microalgae.04.23. SU-1642965

1990: Semenenko VE, Ramazanov Z. (1990) Preparation of radioisotope labeled (carotene and glycerol by growing microalgae. 04.07. SU-1555354

2000: Zakir Ramazanov, Maria del Mar (2000) Method for Obtaining an extract from Ginkgo biloba leaves free of ginkgolic acid, US Patent 6,117,431

AWARDS AND SPECIAL GRANTS

1978:	Graduated with Golden Diploma, the North Caucasian State University, USSR
1981:	Award from the USSR Military Industry for Bioreactors design
1984:	Award from the USSR Academy of Sciences for the best scientific achievement.
1989:	Swedish Royal Science Foundation Grant and Swedish Institute of Natural Science. Umea University
1990-1991:	Ministerio de Educacion y Ciencia, University of Cordoba, Spain
1991:	SAREC, Sweden. Scientific collaboration between Swedish and African Countries (University of Dar Salaam, Marine Science Institute of Zanzibar, Republic of Tanzania.
1994:	National Science Foundation USA, Louisiana State University.
1993:	Grant from Universidad de Autonoma de Madrid, Spain.
1993:	Grant from the University of Las Palmas, Spain.
1994:	Grant from USAID (USA Government) for Development of algal biotechnology in Central Africa: Chad, Cameroon.

INVITED PRESENTATIONS

1977. Cultivation of Protoccocal Microalgae as Nutrition and Fodder protein. 11th FEBS Congress. Copenhagen. Denmark.

1979. Investigation of Carbonic anhydrase activity in Chlorella cells. North Caucasusian Center for High Education. Rostov, USSR

1979. Cultivation of Chlorella cells in thermomineral water. North Caucasian Center of High Education. Rostov, USSR

1981. The perspective of utilization thermo-mineral water for obtaining protein Vitamin- food. In" Low parametric thermomineral energetics". USSR

1983. Oxygen dependence induction of Carbonic anhydras synthesis in Chlorella cells. All-Union Conference of Young Scientists of the USSR. Moscow

1983. Induction of the adaptive CO2 dependent form of Carbonic anhydrase by products of glycolate pathway in Chlorella cells. International Symposium on Regulation of primary and secondary products of phosynthesis. Puschino USSR

1983. Carbonic anhydrase system of photosynthesizing cells: organization, functional role and regulation of synthesis. International Symposium on Regulation of primary and secondary products of photosynthesis. Puschino, USSR

1984. Turkmenian strains of Chlorella. All-Union Symposium "Cultivation and application of microalgae in the national economy of the USSR. Tashkent, USSR

1985. Carbonic anhydrase system of photosynthesizing cells: organization, functional role and regulation of synthesis. International Congress"Kinetics of photosynthetic carbon metabolism in C3 plants. Tallinn, Estonia

1986. Participation of CO_2 dependent Carbonic anhydrase in the regulation of photosynthesis. II All Union Congress of Young Scientist of the USSR. Moscow

1985 Biochemical mechanisms of adaptation of photosynthesizing Chlorella cells to carbon assimilation. All Union Meeting on the Industrial cultivation of micralgae. Ashgabad, USSR

1985. Paramenric control of biosynthesis of the carotene in Dunaliella salina. Meeting on the Commercial cultivation of microalgae. Ashkhabad, USSR

1985. The level of CO2 dependent Carbonic anhydrase as influenced by light intensity and electron transport chain of the chloroplast. Internatational Symposium "Relationship between carbon and nitrogen metabolism during photosynthesis". Pushino,USSR

1986 Regulatory role of the oxygenase function of RUBISCO in the induction of the CO2 dependent form of Carbonic anhydrase in photosynthesizing Chlorella cells.Vth All-Union Biochemical Congress, Kiev, USSR.

1986. Mechanisms of regulation of Carbon assimilation in halophilic algae. III All-Union Congress of Young Scientists of the USSR, Petrosavodsk, USSR

1986. Involvement of photorespiratory products in induction of the CO2 dependent Carbonic anhydrase. International Symposium on Mineral Nutrition and photosynthesis, Varna, Bulgaria

1986. A study of the light induced activatiobn of Carbonic anhydrase in Scenedesmus cells. Interenational Symposium on Mineral Nutrition and photosynthesis. Varna, Bulgaria

1986 Evidence for the participation of Carbonic anhydrase in HCO3 assimilation by Scenedesmus cells. International Symposium on Mineral Nutrition and photosynthesis.Varna, Bulgaria

1988. Regulation of glycerol synthesis in Dunaliella salina. Role of Carbonic anhydrase. Workshop "Biogenesis, structure and function of the photosithetic apparatus in connection with solar energy convertion". Toulboukhin, Bulgaria.

1988. Structural and functional organization of the photosynthetic apparatus of Dunaliella salina under hyperfunction conditions. International Workshop "Biogenesis, structure and function of the photosynthetic apparatus in connection with solar energy convertion". Toulboukhin, Bulgaria.

1988. Effect of CO_2 concentration on photosynthesis, Carbonic anhydrase and Nitrate reductase activity in Dunaliella salina. Workshop "Biogenesis, structure and function of the photosynthetic apparatusin connection with solar energy convertion". Toulboukhin, Bulgaria.

1988. Regulation of glycerol synthesis in halophilic algae. IVth All Union Congress of Young Scientists of the USSR. Petrosavonsk, USSR.

1988 Mechanisms of Carbon assimilation in Spirulina platensis. IVth All Union Congress of Young Scientists of the USSR. Petrosavodsk, USSR

1988. Carbonic anhydrase system of photosynthesizing cells: organization, functional role and regulation of synthesis. VIth Congress of the Federationof European Societies of Plant Physiology (FESPP), Split, Yugoslavia

1988. A study on light-induced Activity of Carbonic anhydrase in Scenedesmus cells. International Meeting on Mineral Nutrition and Photosynthesis. Bulgaria, Sofia

1989. Localozation of Carbonic anhydrase in Dunaliella salina. All-Union Meeting on Physiology of Microorganisms. Tashkent, USSR

1990. Paramentic control of carotene biosynthesis in Dunaliella salina. Vth International Congress of the Applied Algology Society. Jerusalem, Israel

1990. Effect of suboptimal temperature on he content of carotene and lipids in *Dunaliella salina*. International Meeting of the Society Applied Algology. Jerusalem, Israel.

1990. Inorganic carbon concentrationg mechanisms in algal cells. VIIth Congress of the FESPP, Umea, Sweden

1990. The mechanism of light activation of glutamine synthetase isoforms in green algae. VIIth Congress of the FESPP, Umea, Sweden

1990. The role of Nitrogen Nutrition on the regulation of Glutamine Synthetase isoforms in green algae. VIIth Congress of the FESPP, Umea, Sweden

1991. Involvement of photorespiration and glycolate pathway in Carbonic anhydrase induction and inorganic carbon concentration in Chlamydomonas reinhardtii. IV Portuguese-Spanish Biochemistry Congress. Porto, Portugal

1993. The CO_2 concentrating mechanism in algae: the role of the pyrenoid. V1th International Conference on Applied Algology. Czech Republic.

1993. Physiological characterization of high CO_2 requiring mutants isolated from a strain of Chlorella. VIth International Conference on Applied Algology. Czech Republic

1993. Isolation and characterization of high CO_2-requiring mutants of Chlorella. International Botanical Congress, Tokyo. Japan

1995. Induction of a CO_2 concentrating mechanism in starch-less mutants of microalgae. Photosynthesis Congress, FRANCE

1995. The pyrenoid starch sheath formation in high CO_2 requiring mutant of Chlamydomonas reinhardtii. Photosynthesis Congress, FRANCE

1997. The Mystery of the pyrenoid. Umea University, Department of Plant Physiology, Sweden.

1999. Effect of Stress on mental and physical performance. NUMICO, Las Vegas, Nevada

2000. Regulation of Cholesterol and nutrition, New Jersey, Garden State Nutritionals.

2003. Antioxidant activity of edible fruits and flavonoids, Garden of Life, Inc, West Palm Beach, Florida.

2004. Russian Sport: New none-steroidal Sport supplements, Phoenix Lab, Hicksville, New York

2005. Diabetes management. Garden of Life, Inc, West Palm Beach, Florida

SELECTED PUBLICATIONS
(Total 158 Publications)

Arthur Ramazanov, Zakir Ramazanov (2006). Isolation and characterization of a starchless mutant of Chlorella pyrenoidosa STL-P1 with a high growth rate, and high protein and polyunsaturated fatty acid content. Phycological Researech, *in press.*

Zakir Ramazanov, Musa T. Abidov, R. Steven Sikorski, Seifula Roshen, Arthur Z. Ramazanov, Alexander L. Klimenov, Miguel Jimenez del Rio, Oleg V. Kalyuzhin (2006). Freeze-dried Rhodiola rosea extract demonstrates a two-fold advantage in prolonging physical exertion in rats, compared to the spray-dried form. Exp. Biology and Medicine, *in press.*

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Zakir Ramazanov, Brian Appell, Arthur Ramazanov (2003). Stress and Weight Management. ISBN 0-9723437-1-7. Print House Press, Georgia

Zakir Ramazanov, Arthur Ramazanov (2004) Rhodiola rosea, Le orogini e la storia Fitochemica e Farmacologia, Aboca Press, Loc Aboca 20, 52037, Sansepolcro (AR) Italy

Ramazanov Z, Jimenez del Rio M, Ziegenfuss T. (2003). Sulfated polysaccharides of brown seaweed *Cystoseira canariensis* bind to serum myostatin protein. Acta Physiol Pharmacol Bulg. 27(2-3):101-6

Zakir Ramazanov, Brian Appell (2003). Rhodiola Rosea for Chronic Stress Disorder; Monograph. ISBN: 0972343709

Zakir, Dr. Ramazanov, Maria Del Mar Bernal, Dr. Suarez, Nina Anderson, (2003). Stress and Weight Management: Effective Herbal Therapy Using Rhodiola Rosea and Rhododendron Caucasicum. Safe Goods Publishing, CT, USA ISBN: 0972343717

Richard P. Brown, M.D., Patricia L. Gerbarg, M.D., Zakir Ramazanov, Ph.D., D.S. (2002). *Rhodiola rosea:* A Phytomedicinal Overview. *HerbalGram.* American Botanical Council; 56:40-52, USA.

Zakir, Dr. Ramazanov, Maria Del Mar Bernal, Dr. Suarez, Nina Anderson,(1999).New Secrets of Effective Natural Weight management. Safe Good Publishing ISBN: 188482045X.

Carl Germano, Zakir Ramazanov, Maria Del Mar Bernal Suarez (1999). Arctic Root (Rhodiola Rosea) : The Powerful New Ginseng Alternative 01 April, ISBN: 1575664534, Kensington Press, New York

Chkhikvishvili ID, Ramazanov ZM. (2000). Phenol compounds from brown algae and their antioxidant activity. Prikl Biokhim Mikrobiol. 2000 May-Jun; 36(3): 336-8.

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Karlsson J., Hiltonnen Th., Z. Ramazanov & G. Samuelsson (1996). Discovery of an algal mitochondrial carbonic anhydrase: Molecular cloning and characterization of a low-CO2 induced polypeptide in Chlamydomonas reinhardtii. Proceedings of National Academy of Sciences of U.S.A. 93:12031-12034.

Villarejo V, Guillermo Garcia Reina & Z. Ramazanov (1996). Regulation of the low-CO2 inducible polypeptides in Chlamydomonas reinhardtii. Planta, 199: 481-485.

Maria del Pino Plumed, Arsenio Villarejo, Asincion de los Rios, Gulliermo Garcia Reina & Ramazanov Z. (1996). The CO2-concentrating mechanism in a starchless mutant of the green unicellular alga Chlorella pyrenoidosa. Planta, 200: 28-31.

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Zakir Ramazanov, Pedro Sosa, Margaret Henk, Miguel Jimenez del Rio, J L Comez Pinchetti & G. Garcia Reina. (1995). Low C02 inducible protein synthesis in the green alga Dunaliella tertiolecta. Planta, 195: 519-524.

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Plumed MP, Elena del Campo, G.Garcia Reina, & Z. Ramazanov. (1995) The pyrenoid starch sheath formation in high CO2 requiring mutant of *Chlamydomonas reinhardtii.*

Photosynthesis Congress, France

Karlsson J, Ramazanov Z, Hiltonen Th, Gardestrom P and Samuelsson G. (1994) Effect of vanadate on photosynthesis and the ATP/ADP ratio in low CO_2 adapted Chlamydomonas reinhardtii. Planta, 192: 45-50

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Exhibit B.

HPLC chromatogram fingerprint of *Aralia mandshurica/elata* dry **BUD** extract.

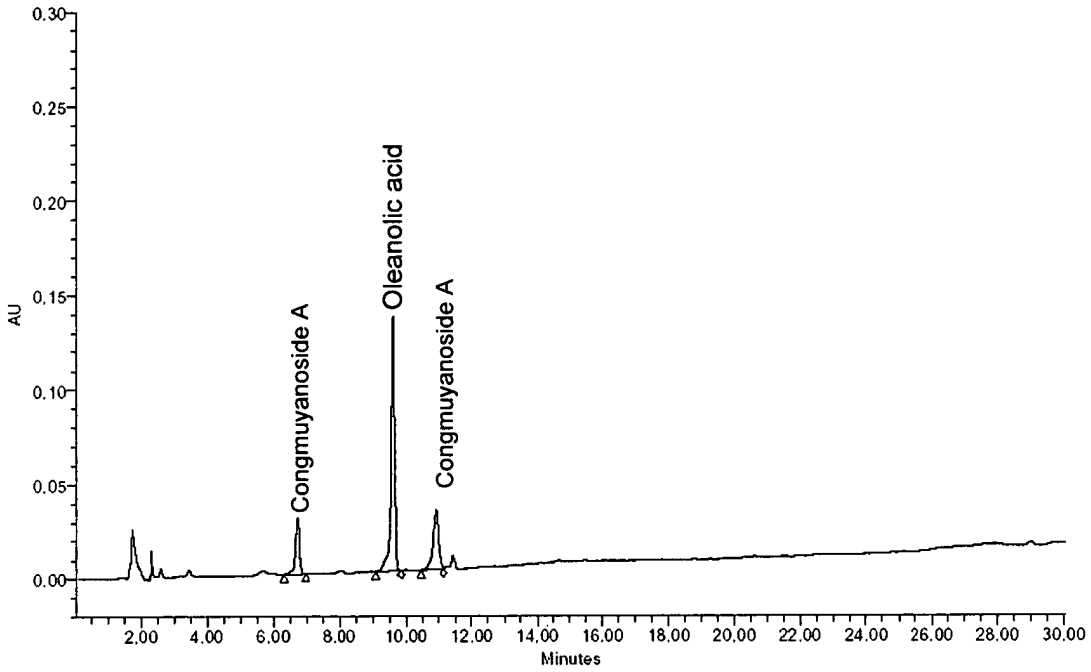


Exhibit C.

HPLC chromatogram fingerprint of *Aralia mandshurica/elata* **ROOT** extract.

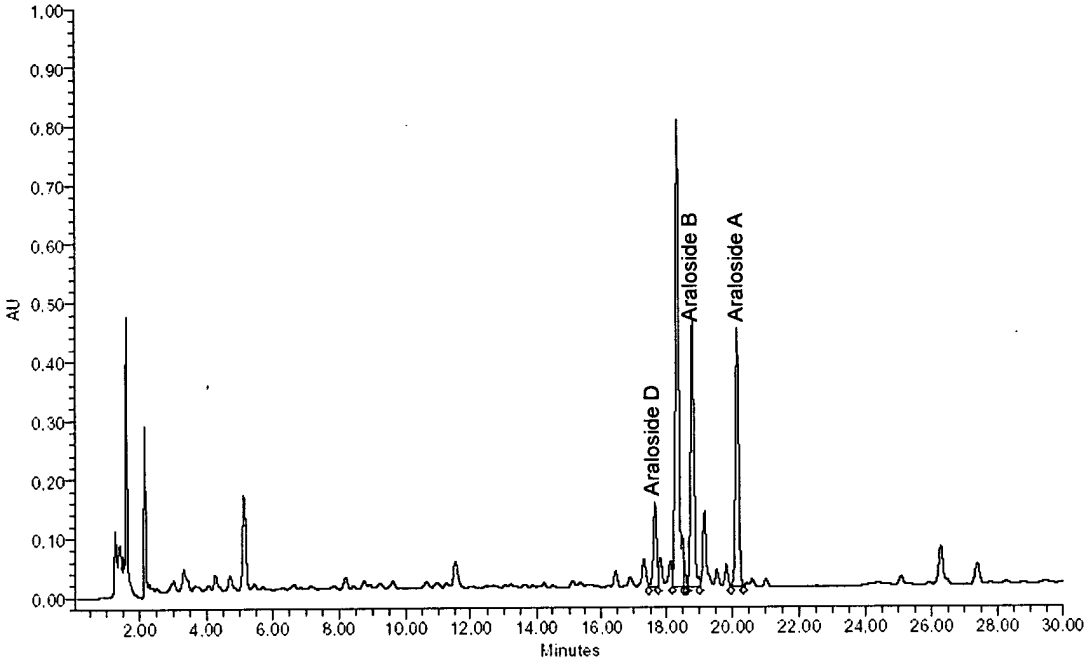
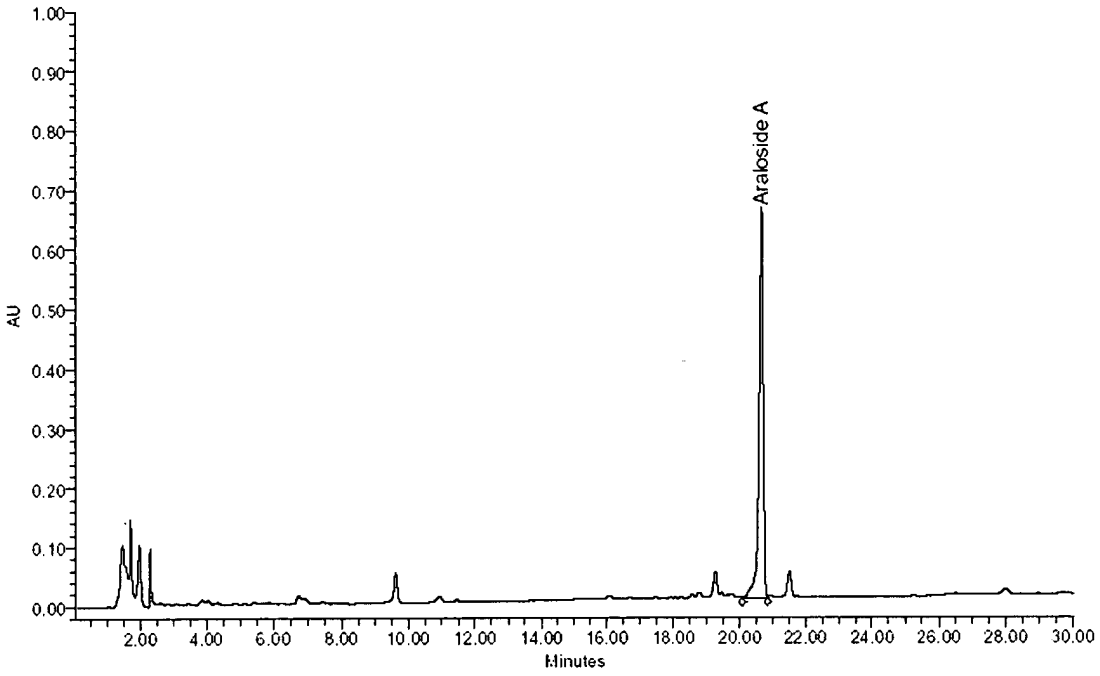


Exhibit D.

HPLC chromatogram of araloside A **REFERENCE** standard.



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Two new saponins from the bud of *Aralia elata* (Miq.) Seem

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Two new saponins, named congmuyanoside A and congmuyanoside B, have been isolated from the buds of *Aralia elata* (Miq.) Seem. Their structures have been determined on the basis of chemical and spectroscopic evidence.

Keywords: Araliaceae; *Aralia elata*; Congmuyanoside A; Congmuyanoside B; Triterpenoidal saponin

1. Introduction

Aralia elata (Miq.) Seem. (Araliaceae) is widely distributed in the northeast of China and Korea. Its root bark has been used as a folk medicine for rheumatism, diabetes and as a tonic in China, Japan and Russia [1]. Triterpenoidal saponins are reported to be the main active principles [2]. The pharmacological action and the chemical components of its bud, which is called "cilaoya", have rarely been reported. In this paper, we describe the isolation and the structure elucidation of two new saponins obtained from the buds of *Aralia elata*.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder. The molecular formula of **1** was determined as $C_{41}H_{66}O_{14}$ by HR-ESIMS, which shows a quasi-molecular ion peak at *m/z* 783.4525 $[M+H]^+$. The IR spectrum indicates the presence of a carbonyl group (1693 cm^{-1}). The ^1H NMR spectrum of **1** exhibits signals characteristic for six methyl singlets at δ 0.93, 0.97, 1.04, 1.06, 1.17, 1.79, one trisubstituted olefinic proton at δ 5.64 and two anomeric protons at δ 4.94 and 5.28. The ^{13}C NMR spectrum of **1** shows signals of a pair of olefinic carbons at δ 122.5 and 145.1, two anomeric carbons of sugars at δ 106.4, 106.5 and a free carboxyl carbon at δ 180.0. The ^1H and ^{13}C NMR spectral data suggests that the aglycone consists of oleanolic acid with a free carboxyl group. Some characteristic chemical

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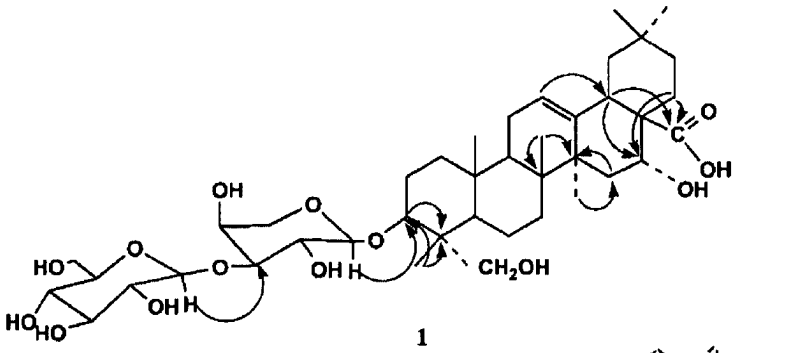
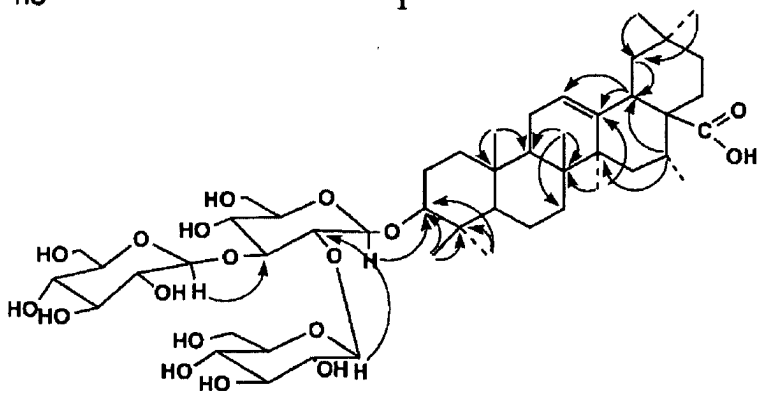
shifts such as δ 64.4 (C-23), 13.7 (C-24), 47.8 (C-5), 81.9 (C-3), 74.8 (C-16), 36.3 (C-15) and 180.0 (C-28) further revealed that compound **1** was a caulophyllogenin glycoside with two sugars attached at C-3. The sugar moieties were identified as arabinose and glucose by co-TLC with authentic samples after acid hydrolysis. The chemical shifts of the sugar moiety in ^{13}C NMR also confirmed the presence of one arabinose and one glucose. The sugar linkages were determined on the basis of the HMBC spectrum. HMBC correlation occurs between a proton signal at δ 4.94 (ara-H-1′) and a carbon signal at δ 81.9 due to C-3 of the aglycone moiety, while an anomeric proton signal at δ 5.28 (glc-H-1″) shows a correlation with a carbon signal at δ 84.3 due to C-3′ of the inner sugar, suggesting glycosylation at C-3 of the aglycone with a glc(1 → 3)-ara moiety. The anomeric configurations of the sugar moieties were determined to be β for glucose and α for arabinose on the basis of the J_{H-H} values (7.5 and 7.2 Hz, respectively). The ^{13}C NMR spectral data of compound **1** is almost the same as that of the known compound congmunoside II [3] (3-*O*-[β-D-glucopyranosyl(1 → 3)-α-L-arabinopyranosyl] caulophyllogenin 28-*O*-β-D-glucopyranosyl ester) except for an additional set of signals due to a glucose, and a highfield shift of C-28 to 175.9 in congmunoside II. All these data confirm that the aglycone of compound **1** is caulophyllogenin, with a glc (1 → 3) sugar moiety at C-3. From the above evidences, the structure of **1** was concluded to be 3-*O*-[β-D-glucopyranosyl (1 → 3)-α-L-arabinopyranosyl] caulophyllogenin. This is a new compound, named here as congmuyanoside A (figure 1).

Compound **2**, a white amorphous powder, shows an absorption band at 1693 cm^{-1} for a carbonyl group in the IR spectrum. Its HR-ESIMS spectrum exhibits a quasi-molecular ion



1



2

Figure 1. Structures and key HMBC correlations of **1** and **2**.

peak at *m/z* 959.5242 $[M + H]^+$, corresponding to the molecular formula $C_{48}H_{78}O_{19}$. The ^1H NMR spectrum of **2** displays signals characteristic for seven methyl singlets at δ 0.81, 1.00, 1.05, 1.06, 1.17, 1.24 and 1.84, one trisubstituted olefinic proton at δ 5.63, and three anomeric protons at δ 4.83, 5.36, and 5.71. The ^{13}C NMR spectrum of **1** shows signals of a pair of olefinic carbons at δ 122.4 and 145.2, three anomeric carbons of sugars at δ 103.9, 104.8, 105.0 and a free carboxyl carbon at δ 180.0. The ^1H and ^{13}C NMR spectral data suggest that the aglycone is oleanolic acid with a free carboxyl group. By comparing the ^{13}C NMR spectral data of **2** with that of oleanolic acid, the signal due to C-16 was shifted downfield by 51 ppm, signals due to C-15 and C-17 were shifted lower field by 8 and 2 ppm, respectively, while the others were almost the same. This indicates that the aglycone of **2** is echinocystic acid with a sugar moiety composed of three sugars at C-3 [4]. The sugar moieties were identified as glucose only by co-TLC with authentic samples after acid hydrolysis. The sugar linkages were determined on the basis of the HMBC spectrum which shows correlation between a proton signal at δ 4.83 (glc-H-1′) and a carbon signal at δ 89.4 due to C-3 of the aglycone moiety, while anomeric proton signals at δ 5.36 (glc-H-1″) and 5.71 (glc-H-1‴) show correlations with carbon signals at δ 88.8 and 79.4 due to C-3′ and C-2′ of the inner sugar, respectively, suggesting glycosylation at C-3 of aglycone with a [glc (1 → 2)]-[glc (1 → 3)] glc moiety. The anomeric configurations of the glucoses were determined to be β on the basis of the J_{H-H} values (7.6, 7.7, 7.7 Hz respectively). From the above evidences, the structure of **2** is concluded to be 3-*O*-[β-D-glucopyranosyl (1 → 2)]-[β-D-glucopyranosyl(1 → 3)] β-D-glucopyranosyl echinocystic acid. This compound has not been reported previously, and is named congmuyanoside B.

3. Experimental

3.1. General experimental procedures

Optical rotations were performed with a Perkin–Elmer 241MC polarimeter. Positive HRESI-MS was taken on a Bruker Daltonics Inc. APEX II FT-ICRMS. IR spectra were measured with a Bruker IFS-55 infrared spectrometer. ^1H and ^{13}C NMR spectra were recorded with a Bruker ARX-300 NMR spectrometer. ESI-MS was taken on a Finnigan LCQ LC-MS analyzer. Column chromatography was carried out on silica gel (Qingdao Haiyang chemical co., Ltd. 200–300 mesh). Preparative HPLC was carried out on a Hitachi LC system with a RI detector and an Alltech ODS column (21.5 × 300 mm, 10 μm). Spots were visualized by spraying with ethanol–10% H_2SO_4 and heating (110°C; 5 min).

3.2. Plant material

Buds of *Aralia elata* collected from Liaoning Province of China in August 2000 were taxonomically identified by Professor Sun Qishi of Shenyang Pharmaceutical University. A voucher specimen has been deposited at the School of Traditional Chinese Material Medica, Shenyang Pharmaceutical University.

3.3. Extraction and isolation

The air-dried buds (10 kg) were first extracted with 75% ethanol (3 ×) under reflux. The combined solutions were then concentrated *in vacuo*, and subsequently subjected to

Table 1. ^{13}C NMR data of **1** and **2** (ppm, in C_5D_5N).

	1		*2*	
Carbon	δ_C *(ppm)*	δ_H *(ppm) (J in Hz)*	δ_C *(ppm)*	δ_H *(ppm) (J in Hz)*
1	38.9		38.8	
2	26.3		26.6	
3	81.9	4.28	89.4	3.29d (7.9)
4	43.6		39.6	
5	47.8	1.71	56.0	
6	18.2		18.6	
7	32.9		32.9	
8	40.0		40.0	
9	47.4		47.2	
10	37.1		37.0	
11	23.9		23.9	
12	122.5	5.64 (br.s)	122.4	5.63 (br.s)
13	145.1		145.2	
14	42.2		42.2	
15	36.3	2.34	36.3	
16	74.8	5.21 (br.s)	74.8	4.85 (br.s)
17	48.9		49.0	
18	41.5	3.62d (12)	41.5	3.61d (11.4)
19	47.3		47.4	
20	31.1		31.1	
21	36.3		33.4	
22	33.4		33.4	
23	64.4		28.1	1.24s
24	13.7	0.93s	16.8	1.06s
25	16.3	0.97s	15.6	0.81s
26	17.6	1.06s	17.5	1.00s
27	27.2	1.79s	27.3	1.84s
28	180.0		180.0	
29	33.4	1.04s	33.4	1.05s
30	24.8	1.17s	24.8	1.17s
3-O-sugar	Arabinose		Glucose	
1′	106.5	4.94d (7.2)	105.0	4.83d (7.6)
2′	72.1	4.56	79.4	4.38
3′	84.3	4.07	88.8	4.24
4′	69.3	4.34	70.1	4.02
5′	67.1	3.52, 4.13	77.8	3.85
6′			63.5[a]	4.52[a]
	glucose			
1″	106.4	5.28d (7.5)	103.9	5.71d (7.7)
2″	75.8	4.0	76.5	4.08
3″	78.4	4.24	78.7	4.24
4″	71.7	4.18	71.7	4.18
5″	78.7	3.98	77.7	3.89
6″	62.8	4.51	62.4[a]	4.44[a]
1‴			104.8	5.36d (7.7)
2‴			75.5	4.05
3‴			78.7	4.21
4‴			72.6	4.15
5‴			78.7	4.21
6‴			62.6[a]	4.28[a]

[a] Signals in each column may be interchangeable. All signals were assigned by ^1H and ^{13}C NMR, HMQC and HMBC.

macroporous resin D101 column chromatography, eluting with 60% EtOH. The solution was then evaporated to dryness under vacuum to afford a residue (150 g) that was chromatographed on silica gel with $CHCl_3$–MeOH (in gradient) to give 12 fractions (fr. 1–12). Fraction 7 (23 g) was further separated by silica-gel column chromatography with a solvent system of EtOAc–EtOH–H_2O (9:1:0.1) to give **1** (80 mg). Fractions 11 and 12

(22 g together) were repeatedly chromatographed on silica gel with $CHCl_3$–MeOH–H_2O (85:15:10) to obtain 5 fractions, of which fraction 1 (1.44 g) was separated with preparative RP-18 HPLC (MeOH–H_2O 1:1) to yield **2** (62 mg).

Compound 1. White amorphous powder; $[\alpha]_D^{25}$ +21.8 (MeOH, *c* 0.11); IR (KBr) (νcm^{-1}): 3426, 2944, 1693, 1448, 1386, 1258, 1077, 1035, 786; ^1H, ^{13}C NMR data see table 1. Positive ESI-MS: *m/z* 805, 783, 621, 347, 282, 279; HR-ESIMS *m/z* 783.4525 (calcd for $C_{41}H_{67}O_{14}$, 783.4425).

Compound 2. White amorphous powder; $[\alpha]_D^{25}$ +13.3 (MeOH, *c* 0.06); IR (KBr) (νcm^{-1}): 3408, 2944, 1693, 1387, 1159, 1078, 637. Positive ESI-MS: *m/z* 958, 796, 778, 634, 616, 486, 455, 324; ^1H, ^{13}C NMR data see table 1. HRESIMS: *m/z* 959.5242 (calcd for $C_{48}H_{79}O_{19}$, 959.5210).

Acid hydrolysis of 1 and 2. Each saponin was added on a silica gel TLC plate that was then kept in HCl moisture at 80°C for 2 h, and was then taken out and dried. The plate was developed in a solution of $CHCl_3$–MeOH–H_2O (65:35:10 lower layer) together with authentic samples of sugar, R_f: L-arabinose, 0.69; D-glucose, 0.61.

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Exhibit F.

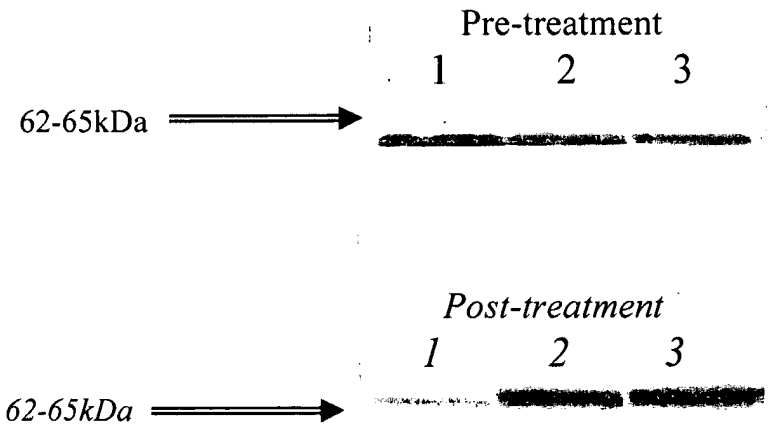
Results: This radiograph shows comparison of the SDS-PAGE and Western Blot Immunoelectrophoresis of pre-treatment and post-treatment adipocytes probed with perilipin antiserum. Scanning Densitometrical analysis of immunoreactive proteins indicates:

a) The proteins in adipocytes probed with anti-perilipin antibody showed strong reaction with 62-65 kDa protein,

b) There were no significant differences in the amount of perilipin protein in adipocytes in baseline (pre-treatment adipocytes),

c) In post-treatment adipocytes of patients who received the ARE dose, the perilipins protein content of adipocytes was reduced from 60.7 ± 10.6 arbitrary units (*a.u.*) before the trial to app. 44.3 ± 12.5 *a.u.* after the trial ($p < 0.05$).

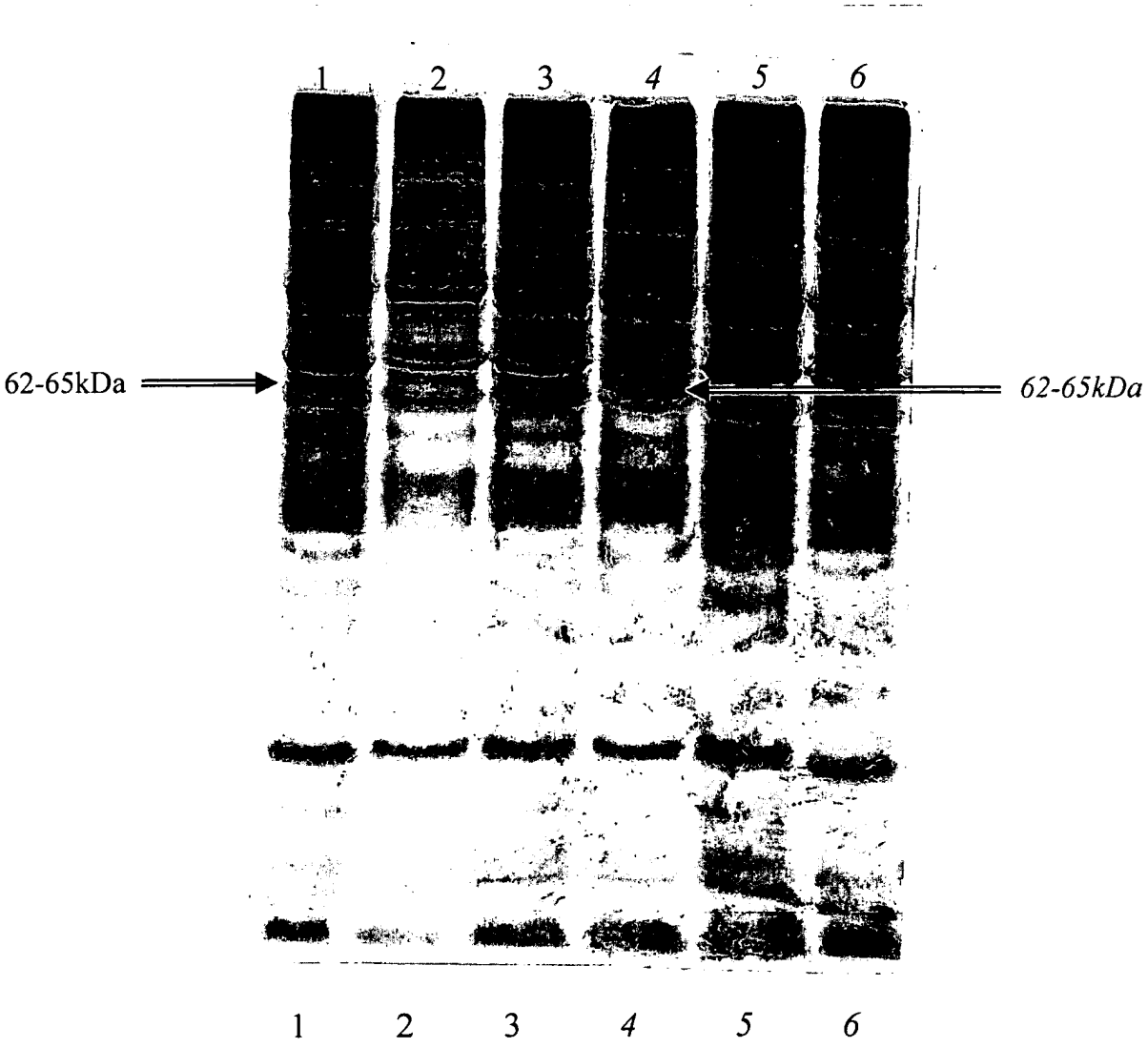
d) No significant perilipins protein changes (from 60.2 ± 12.5 *a.u.* to 62.1 ± 11.2 *a.u.)* were observed in the ABE dose or placebo groups.



SDS-PAGE and Western blot immunoelectrophoresis of pre-treatment and *post-treatment* adiposities probed with antibody raised against purified human perilipin polypeptide. Lane 1, 2 3: samples of adipocytes of patients from the ARE, the ABE and the Placebo groups

Baseline	Aralia bud extract (ABE)	Aralia root extract (ARE)
60.7±10.6	60.2±12.5	44.3±14.5

Exhibit G.



SDS-PAGE authoradiographs of the pre-treatment and *post-treatment* adipocytes subjected to ^{35}S-methionine pulse-chase labeling. Lanes 1, 2, 3: pre-treatment samples of adipocytes from patients in the ARE, ABE and placebo groups, respectively. Lanes *4, 5, 6*: *post-treatment* samples of adipocytes from patients in the ARE, ABE, and placebo groups, respectively.